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SUBJECT OF INVESTIGATION

PHYSICAL AND BIOCHEMICAL STUDIES ON THE MICROSOMES AND RIBOSOMES

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THE ABSTRACT OF THE FINAL REPORT NO. 4

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In this investigation, it has been intended to obtain more direct evidence on the nature of 50 S and 60 S component and to study on the large and small subunit by the sedimentation analysis as well as by the electron microscopic observation.

1. Sedimentation analysis either by ultracentrifugation or by sucrose density gradient centrifugation has shown that the 50 S and the 60 S components are both composed of the large and the small subunit.

2. It was found that the small subunit is released from the 50 S and 60 S component in the solution containing less than $1 - 2.5 \times 10^{-5}$ M Mg ion, while in the solution containing more Mg ion, no such release was observed.

3. The small and the large subunit were isolated in the presence of Mg and the association between the same and the different kind of ribosomal subunit was examined.

4. These components, which were isolated by the sucrose density centrifugation, were observed by an electronmicroscope using shadow casting method. All the particles were spherical except the small subunit which appeares as a flattened disc. The observed diameters of these particles were clearly larger than those for the unhydrated spherical molecules calculated from their molecular weights but were smaller than those of hydrohynamically equivalent spheres calculated from the molecular weight and sedimentation coefficient.

PHYSICAL AND BIOCHEMICAL STUDIES
ON
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Final Resport No. 4

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CONTENTS PAGE

APPENDIXES

I. THE PURPOSE OF THIS INVESTIGATION

It has been well established that E. coli ribosomes (70 S) are composed of a large (50 S) and a small subunit (30 S) which has two-thirds and one-third of the molecular weight of the 70 S particles respectively (Tissieres et al, 1959).

Liver ribosomes are, however, dissociated first into the two intermediate components with sedimentation rate of 50 S and 60 S and by further removal of Mg, into a large (47 S) and a small (32 S) subunit similar to E. coli ribosomes. From the molecular weight determination, it was suggested that, in the intermediate stage, the small subunit probably exists as a dimer, thus overlapping the sedimentation peak of the large subunit (Tashiro & Siekevitz, 1965a; Tashiro & Ypahntis, 1965).

In view of the importance of ribosome structure in protein synthesis, we have intended to study further in detail on the dissociation of liver ribosomes and the physicochemical properties of these various dissociation products.

1. First the intermediate 50 S and 60 S components were isolated by sucrose density gradient centrifugation and their sedimentation properties were analysed under various experimental conditions either by analytical centrifugation or by the sucrose density centrifugation.

2. The small and the large subunit were isolated by the sucrose density gradient centrifugation and the association of these subunits was examined in the presence of various amount of Mg.

3. These isolated components were observed by an electron-microscope using shadow casting techniques in order to get some information about their molecular dimension as well as the molecular configuration of these particles.

II. EXPERIMENTALS

1. Preparation of Hepatic Ribosomes.

Ribosomes were prepared from guinea pig liver as previously described (Tashiro & Siekevitz, 1965a), resuspended in solution C, at a concentration of 10 mg. per ml. and stored in the cold.

2. Dissociation of Ribosomes to the 50 S and 60 S Components.

Ribosomes were dialysed at 0° against solution E for 24 - 36 hours changing the outside solution several times, usually twice (Tashiro & Siekevitz, 1965a). Dissociation of the ribosomes to the 50 S and 60 S components were confirmed by an analytical centrifuge.

3. Complete Dissociation of Ribosomes to the Large and the Small Subunit..

Ribosomes were dissociated by EDTA treatment usually at a concentration of 2.5 μ mole EDTA per mg. ribosomes and then the subunits ere isolated by the sucrose density gradient centrifugation (Tashiro & Siekevitz, 1965b).

4. Sucrose Densiy Gradient Centrifugation.

For preparative purpose, a linear sucrose gradient from 5 to 20 % (30.5 ml) were set up and a Hitachi RPS-25 roter were used for the centrifugation. The gradient was fractionated into about 30 tubes after run and their optical density was read by a Hitachi spectrophotometer after appropriate dilution. For analytical purpose, a linear sucrose density gradient from 5 to 20 % (4.6 ml.) was set up, and a Hitachi RPS-40 roter was used for centrifugation. The gradient was fractionated into about 30 tubes and their optical density was determined by the same spectrophotometer using microcubette. The minimum amount of the solution required for the measurement was about 0.3 ml.

Solution C: 0.001 M $MgCl_2$, 0.001 M tris-HCl buffer, pH 7.6

Solution D: 0.002 M $MgCl_2$, 0.05 M KCl, 0.001 M tris-HCl buffer, pH 7.6.

Solution E: 0.05 M KCl, 0.001 M tris-HCl buffer, pH 7.6

5. Analytical Centrifugation.

Sedimentation analysis were carried out in the cold (3 to 5°) with a Spinco model E analytical centrifuge, using a UV absorption optical system, usually at a ribosome concentration of 0.8 optical density unit per cm., which correspond about 60 µg ribosomes per ml. UV photographic films were traced by Spinco model R analytrol with the microdensitometer attachment to calculate the sedimentation coefficient as well as the relative proportion of each component. Schlieren optical system was used to analyse more concentrated solution, such as the confirmation of the dissociation of ribosomes to the 50 S and 60 S component.

6. Electronmicroscopic Observation.

Samples for electronmicroscopic observation were first diluted to an appropriate concentration, fixed with 6.25 % glutaraldehyde (Sabatini, Bensch & Barrnett , 1963) and then put onto the specimen grids, covered with carbon coated collodion membrane. Excess samples were soaked with a filter paper and washed twice with distilled water. Polystyrene latex spheres with an average diameter of 880 Å were added to the preparations to provides an internal standard for measuring the shadow-to-height ratio. Pt-Pd alloy (8 : 2) were evaporated at a shadow-to height ratio of about 5 : 1 from the distance of - 7 cm, and observed by a Hitachi HU-11 electronmicroscope at a direct magnification of 10, 000 - 20,000 ×.

III. RESULTS OBTAINED

1. Sedimentation Analysis of the Intermediate 50 S and 60 S Components.

This experiment is intending to determine what the ribosomal constituents of the intermediate 50 S and 60 S components are.

The 50 S and 60 S components, isolated by sucrose density gradient centrifugation in solution E, were either diluted with solution E, or dialysed against solution E, and/or treated with EDTA, and then analysed either by an analytical centrifuge or by the sucrose density gradient centrifugation.

The former analysis showed that the isolated 50 S and 60 S components are always composed of the two components with sedimentation coefficient of 25 - 30 S (27.5 ± 1.5, average of 17 runs) and 40 - 45 S (43.0 ± 1.9, average of 17 runs) respectively. Addition of EDTA produced any remarkable change neither in the proportion nor in the sedimentation coefficients of these two components. These values are definitely smaller than the sedimentation coefficients of the large and the samll subunit which have been reported previously (Tashiro & Sekevitz, 1965a). Therefore, the small and the large subunits were isolated by the same procedures and their sedimentation coefficients were determined under exactly the same conditions (concentration of ribosomes is - 60 µg per ml.). The $S_{20,w}$ values were found to be 43.1 (average of three runs) and 27.2 ± 1.5 (average of six runs) respectively. It is safely concluded, therefore, that the slower component of 25 - 30 S is the small subunit, while the faster component of 40 - 45 S is the large subunit. Probably these subunit particles were hydrated more as they are isolated in the solution containing no Mg or very low concentration of Mg, and also diluted very much with the same solution.

It is now evident that the 50 S and 60 S component are both composed of the two subunits. The average weight ratio of the large to the small subunit for the 50 S component is 1.5 (1.38, 1.35, 1.67) while that for the 60 S component is 3.1 (3.32, 3.56, 2.44). These data clearly show that the 50 S component contains more small subunit, while the 60 S component contains more large subunit than the undissociated ribosomes of which corresponding value was 2.3 ± 0.2.

2. What Factors are Responsible for the Release of the Small Subunit from the Intermediate 50 S and 60 S Components?

One possibility is the dilution effect of the ribosomes themselves, because, in the reversible monomer-dimer dissociation association reaction, dilution may prefer to the production of monomer. Another possible factor is decrease in the Mg concentration of the suspending media, because, by dilution with solution E, Mg itself is also diluted. If the latter factor is predominant, what concentration of Mg ion is necessary for the prevention of the release of the small subunit?

Ribosomes were first dissociated to the intermediate stage by the conventional dialysis against solution E, and then the 50 S and 60 S component produced were isolated in the sucrose density gradient containing various concentration of Mg ion, diluted and/or dialysed against the same solution and analysed again by the sucrose density gradient centrifugation in the presence of the exactly same amount of Mg ion. It is shown that the Mg concentration which prevents the release of the small subunit from the 50 S or 60 S component is rather critical: If the Mg concentration is more than 5×10^{-5} M, release of the small subunit was completely prevented, while if it is less than 10^{-5} M, its release was inevitable.

3. Association Between the Same and the Different Kind of the Isolated Subunit Particles.

The small and the large subunits were isolated by the sucrose density gradient centrifugation in the presence of 10^{-3} M and 10^{-4} M Mg respectively. These subunits were incubated separately for several hours in the cold in the presence of 10^{-3} M, 2×10^{-3} M, 2.5×10^{-3} M, 3×10^{-3} M or 5×10^{-3} M Mg, and then analysed by the sucrose density gradient centrifugation in the presence of the same concentration of Mg respectively (when the Mg concentration is less than 10^{-3} M, no association was observed). In the presence of more than 2×10^{-3} M Mg, several discrete boundaries with larger sedimentation coefficient than monomer boundary were observed: For the small subunit, they were 46 ± 2 S, 55 ± 1 S and 74 ± 3 S and for the large subunit, they were $62 \pm .3$ S, 70 ± 1 S and 80 ± 3 S respectively. These discrete boundaries probably correspond to the dimer, trimer or tetramer of the subunit particles respectively, though we can not neglect the possibility that they are also produced by the configurational change of the subunit particles.

As the next experiment, the large and the small subunit, isolated in the presence of 10^{-3} M and 10^{-4} M Mg respectively,

were mixed at the weight ratio of - 2, and, after incubation for several hours in the cold in the presence of 10^{-3} M, 2×10^{-3} M, or 3×10^{-3} M respectively, the association between the small and the large subunit particles were examined by the sucrose density gradient centrifugation in the presence of the same concentration of Mg. Besides the peaks corresponding to the small and the large subunit particles, discrete boundaries were observed at the 57 ± 2 S, 68 ± 3 S and 93 S position. These boundaries are however, not remarkable as compared with the discreted boundaries which were produced by the association of the small or the large subunits themselves. It may be concluded that, under the present experimental conditions, no preferential associat on between the small and the large subunit particles was observed.

4. Electronmicroscopic Observation of the Undissociated Ribosomes and 50 S Component, the Large and the Small Subunits.

a. Undissociated ribosomes.

Fig. 1 a show the electronmicrograph of undissociated ribosomes. As this material was not fractionated by the sucrose density gradient centrifugation, it is observed that many of the ribosomes exist as polymers or aggregates. Some of these aggregated particles are connected with a filamentous material approximately 40 Å in diameter. Occasionally the particles show a substructure as though they were composed of two or more subparticles.

The diameter of the monomer ribosomes given in Table 1 is not only larger than that of E. coli ribosomes reported by Hall and Slayter (1960); 260 × 170 Å, after correction for width of shadow metal, 200 × 170 Å, or that of Novikoff hepatoma ribosomes reported by Kuff and Zeigel (1961); 260 - 280 × 180 Å, but also those of hepatic ribosomes reported from our laboratory (Inoue, Tashiro, Shimizu & Masumura, 1961); 210 × 150 - 160 Å. after correction for width of shadow metal, 160 Å × 160 - 150 Å.

b. 50 S component.

The sample for the 50 S component was isolated by the sucrose density gradient in the absence of Mg (solution E). Their electronmicrograph (Fig. 1 b) and the dimensions of the particles (Table 1) clearly show that the particles found in this component is definitely smaller than the monomer ribosomes, thus excluding the possibility that it is an unfolded form of the monomer particles as discussed in the previous paper (Tashiro & Siekevitz, 1965a). Besides the

particles of - 280 Å, in diameter, existence of smaller particles about 210 Å in diameter was also noted, which composed of about 20 % of the total population in this preparation. This is not surprising, because ultra-centrifugal analyses show that dimer of the small subunit in the 50 S component isolated in the absence of Mg is already at least partially dissociated as previously described, and further fixation either by formaldehyde or glutar-aldehyde rather facilitates the dissociation (Sabatini & Tashiro, to be published). Most of the larger particles in this 50 S preparation, therefore, probably represent the large subunit, while the smaller particles may be the small subunit, though the possibility that some of the large particles are dimer of the small subunit cannot be neglected.

It is interesting that the large subunit particles have frequently a tail or tails in their molecules which look like bacteriophage. The frequency of appearance of the large subunit particles with such tail(s) was approximately 30 % in this sample. It is not certain whether they are the particles on the way to complete unfolding, thus the tail(s) representing free end(s) of partially unfolded ribo-nucleoprotein chain or they do represent a structural component of the large subunit particles.

c. Large subunit (47 S).

The large and the small subunits were isolated by the sucrose density gradient centrifugation as described previously.

It has been postulated that the 47 S component is the large subunit with much more hydration water than the monomer particles. This suggestion seems to be supported also by the present electronmicroscopic observation (Fig. 1 c). They are, in general, spherical in shape, but they seem much more easily deformable than the large subunit in the 50 S component. The particles with a tail or tails are more frequent and some of them are deformed to rod like molecules.

d. Small subunit (32 S).

It is shown that the particles in this fraction have much flattened structure than the other particles and also there exist a considerable amount of aggregates. The latter property of the small subunit has been observed by sedimentation analysis (Tashiro & Yphantis, 1965). It is also noted that the particles in this fraction usually do not have tail.

IV. DISCUSSION

1. Sedimentation Studies.

Dissociation of hepatic ribosomes are characterized by the existence of an intermediate stage of dissociation where 50 S and 60 S component present without releasing any - 30 S component. It has been suggested that, in the intermediate stage, the small subunit exists as polymer, probably as dimer, thus overlapping the sedimentation peak of the large subunit (Tashiro & Siekevitz, 1965a; Tashiro & Yphantis, 1965). In this experiment, direct evidences that the small subunit does exist in the isolated 50 S component as well as in the 60 S component are presented.

It was pointed out that the factor which controls the release of the small subunit either from the 50 S or the 60 S component is exclusively the concentration of Mg ion, and more than 5×10^{-5} M Mg is necessary for the stability of the hypothetical dimer of the small subunit, the small subunit being released in the solution E containing less than 1×10^{-5} Mg.

Probable existence of a dimer of the small subunit in the 50 S and 60 S component suggest that the small subunit may have strong affinity to associate each other to make dimer in the presence of Mg. Experiments using isolated small subunit, however, have failed to show such a selective association of the small subunit, the isolated large subunit being also polymerized in the presence of the same amount of Mg necessary for the polymerization of the small subunit. This suggests strongly the irreversible character of the dissociation reaction of the small subunit, though we do not yet know what factor is responsible for this irreversibility of the reaction.

It has been repeatedly reported that ribosomes from various sources are dissociated reversibly into large and small subunit particles (Chao & Schachman, 1956; Ts'o, Bonner & Vinograd, 1958; Tissieres et al, 1959). Most of these experiments, however, have been carried out without using isolated subunits. Thus possibility remains that not all of the reconstituted particles are the recombinant of the large and the small subunit but some of them are the polymer of either the small or the large subunit particles themselves. In the present experiment, we have failed, under the present experimental conditions, to show any preferential association between the

large and the small subunit particles, compared with the interaction between the same kind of subunit particles. There are possibilities that some factors which are necessary for the association between the large and the small subunit are lost during the isolation procedures of these subunits, or these subunit particles are suffered from some irreversible configurational change, thus losing their ability to interact each other.

Huxley and Zubay (1960) have reported that the large subunit (50 S) of E. coli ribosomes is dimerized to 81 S particles. The dimerization of small subunit, however, has never been reported. Morimoto (to be published) working with E. coli ribosomes, has recently found that it is not the large subunit but the small subunit which has an ability to make dimer, and that the so-called derived subunit of E. coli ribosomes contains dimer of the small subunit. It is quite interesting that dimer of the small subunit is observed not only in hepatic ribosomes but also in E. coli ribosomes.

2. Electronmicroscopic Observation.

The diameter of the undissociated ribosomes monomer is clearly larger than that for the unhydrated molecules calculated from the molecular weight (Tashiro & Yphantis, 1965) and partial specific volume of 0.66 (Hamilton & Petermann, 1959) but it was much smaller than that of hydrodynamically equivalent sphere calculated from the molecular weight and sedimentation coefficient (Tashiro & Siekevitz, 1965a), probably because the particles were shrunk considerably during drying process (Williams, 1953; Hart, 1962). Further, disagreement of the diameter of ribosome monomer determined in this experiment with that determined in the previous experiment suggests that the glutaraldehyde fixation is, in some degree, effective in preventing the shrinkage of ribosomes during drying process.

The hydration probably increases further as the dissociation of the ribosomes proceeds as calculated from the molecular weight and S value, and it is not surprising that the discrepancy between the observed dimension of the particles and the expected diameter becomes even more remarkable. In the case of the large subunit, however, deformation of the particles like tail formation or increase in axial ratio might also contribute to the decrease in the sedimentation coefficient.

Such situations make it very difficult to expect the

agreement of the observed dimensions of the particles with the hydrodynamically equivalent sphere. We could, however, convincingly conclude from these electronmicroscopic observations that (1) the 50 S component is not unfolded monomer, but a dissociation product of the monomer and (2) both the large and the small subunit particles are remarkably swollen and accordingly much more hydrated than the original monomer.

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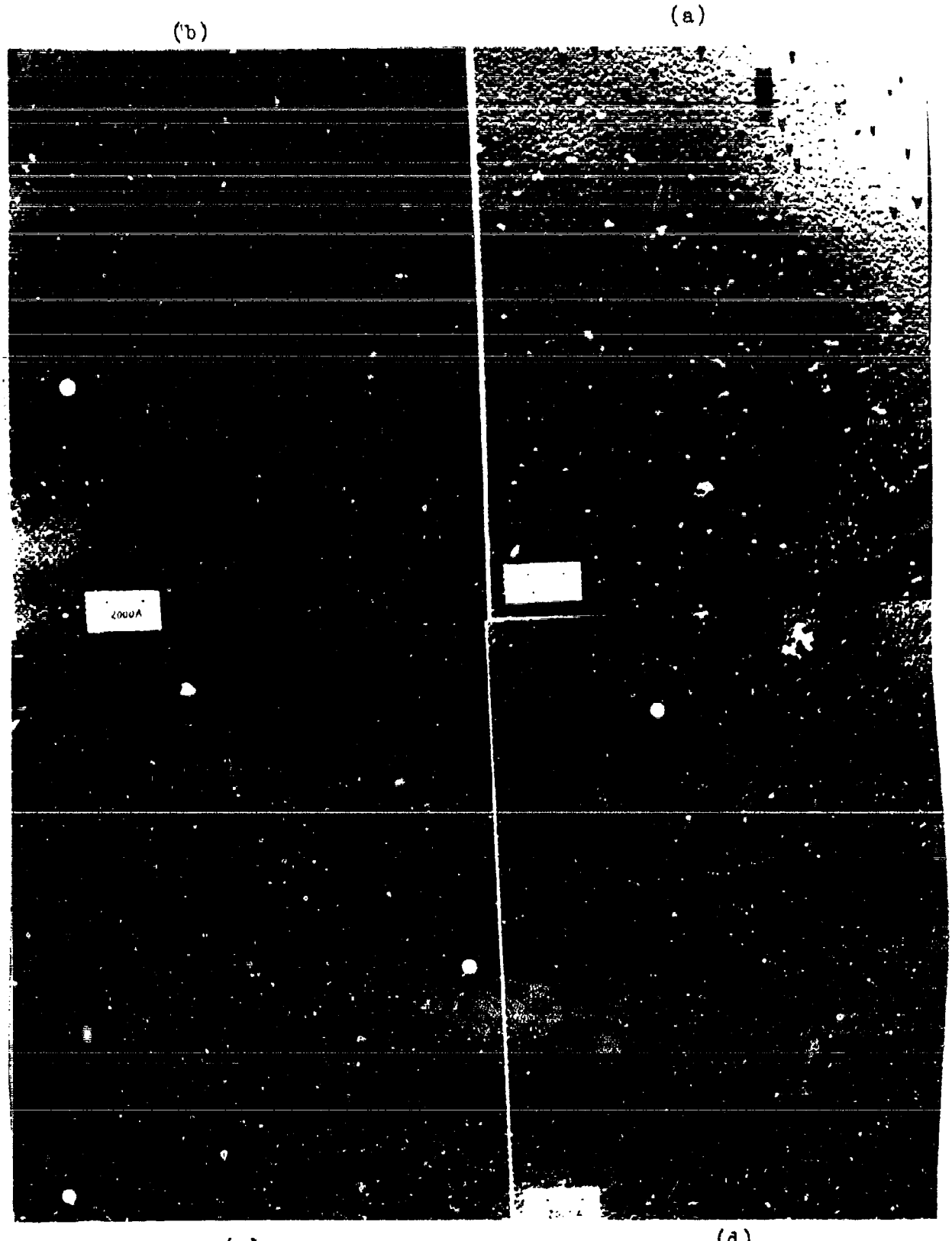
APPENDIX A

Table 1. Physicochemical properties of hepatic ribosomes.

Component	Molecular weight $\times 10^6$	Sedimentation coefficient in Svedbergs $S_{20,w}$ *	Observed particle		Calculated diameter in A	
			width**	height	Unhydrated sphere	Hydrodynamically* Equivalent sphere
Monomer	5.0_0	77	320 ± 9 % (260)	200	220	390
50 S						
Large particles	---	50	280 ± 10 % (220)	140	190	400
Small particles	---	50	210 ± 12 % (150)	90	---	---
Large subunit	3.3_3	47	290 ± 12 % (230)	130	190	430
Small subunit	1.6_7	32	280 ± 10 % (220)	70	150	310

* As described in the text and in the previous paper (Tashiro & Siekevitz, 1965a), sedimentation rates of the large and the small subunits depend very much upon the various experimental conditions. For the calculation of the diameter of hydrodynamically equivalent sphere of the large and the small subunit 47 S and 32 S were conventionally used in this table.

** Figures in the bracket is the corrected diameter of the particles assuming that the contribution from the cap of shadowing metal is 60 Å.


(b)
(a)
2000A
(c)
(d)

APPENDIX B

Fig. 1. Electronmicrographs of the hepatic ribosomes.

(a) Undissociated ribosomes

(b) 50 S component

(c) Large subunit

(d) Small subunit

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13. ABSTRACT

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1. Sedimentation analysis either by ultracentrifugation or by sucrose density gradient centrifugation has shown that the 50 S and the 60 S components are both composed of the large and the small subunit.

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particles were spherical except the small subunit which appeared as a flattened disc. The observed diameters of these particles were clearly larger than those for the unhydrated spherical molecules calculated from their molecular weights but were smaller than those of hydrodynamically equivalent spheres calculated from the molecular weight and sedimentation coefficient. (Author)